Identification of a set of genes expressed during the G0/G1 transition of cultured mouse cells

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To identify previously undetected genes that may be involved in the transition from a resting state (G0) to a proliferative state (G1) of mammalian cells, we set out to isolate cDNA clones derived from mRNAs that appear in serum-stimulated cells in the absence of protein synthesis. A λ cDNA library was prepared using poly(A)$^+$ RNA from BALB/c 3T3 cells that had been brought to quiescence and subsequently stimulated with serum in the presence of cycloheximide. Approximately 50 000 recombinant phage plaques were screened, and 357 clones were isolated that hybridized to probes derived from stimulated-cell RNA but not to probes from resting-cell RNA. Cross hybridization analysis showed that four RNA sequence families account for ~90% of these clones. One of the clones hybridized to an actin probe; none hybridized to any of 13 oncogene probes tested. Five different RNAs that appear to be previously uncharacterized have been further analyzed. These RNAs accumulate and decay rapidly following stimulation by serum or purified growth factors, or by a tumor promoter, and they are superinduced by serum in the presence of cycloheximide. Three of the RNAs could be enriched by hybridization to cDNAs and translated *in vitro*, yielding proteins of ~43, 40 and 35 kd, respectively.

Key words: cell cycle/gene expression/growth factors/growth-related mRNAs

Introduction

Progression through the animal cell cycle is regulated primarily during the G1 phase (for reviews, see Pardee *et al.*, 1978; Baserga, 1985). Genetic and biochemical evidence suggests that synthesis of new mRNAs is required for progression through G1: α-amanitin blocks cells in G1 (Waechter *et al.*, 1984), and a temperature-sensitive mutant cell line that is arrested in G1 at the non-permissive temperature is defective in RNA polymerase II (Rossini *et al.*, 1980; Shales *et al.*, 1980). Protein synthesis in early G1, possibly directed by newly transcribed messages, is also required for cell growth (Schneidrman *et al.*, 1971; Pardee *et al.*, 1978). Moreover, it has been estimated that in mouse fibroblasts, 3% of the mRNA species in logarithmically growing cells are absent in non-growing cells (Williams and Penman, 1975). Several laboratories have thus sought to examine G1-specific RNAs synthesized by cells in response to treatment with serum or purified growth factors (Foster *et al.*, 1982; Cochran *et al.*, 1983; Linzer and Nathans, 1983; Matrisian *et al.*, 1985a, 1985b), or RNAs made in mutant cells conditionally blocked at the G1 phase (Lee *et al.*, 1983; Hirschhorn *et al.*, 1984).

Mouse BALB/c 3T3 cells in culture can be brought to a quiescent state (G0) by growth to confluence and by serum deprivation. These cells can be subsequently stimulated to re-enter G1 by the administration of serum or purified growth factors (Pardee *et al.*, 1978). Although there has been some controversy as to whether G0 is a distinct physiological state or merely a different manifestation of the G1 phase, evidence has been accumulating supporting the former notion (Baserga, 1985). For instance, the c-*myc* proto-oncogene is not expressed in quiescent cells (Kelly *et al.*, 1983) but is constitutively expressed in all phases of cycling cells (Thompson *et al.*, 1985). More recently, the c-*fos* proto-oncogene has been found to be expressed transiently during the G0/G1 transition (Greenberg and Ziff, 1984; Kruijer *et al.*, 1984; Müller *et al.*, 1984), further suggesting that important growth regulatory events can occur during this transition and that these events involve the accumulation of mRNAs encoding regulatory proteins.

In an effort to identify and characterize genes that may play a role in growth control, we set out to identify genes that are expressed at specific times following the stimulation of resting BALB/c 3T3 cells by serum or growth factors (Linzer and Nathans, 1983; Kahana and Nathans, 1984). We describe here the identification of a set of genes whose expression, in the form of mRNA, occurs during the early phase of the G0 to G1 transition. To identify such genes a cDNA library was prepared from poly(A)$^+$ RNA from cells stimulated to enter the G1 phase with 20% serum (MEM-20) in the presence of cycloheximide. Our purpose in inhibiting protein synthesis with cycloheximide was 2-fold: first, to limit new mRNAs to those that do not require *de novo* protein synthesis for their accumulation (analogous to viral 'immediate early' RNAs); and second, to amplify mRNAs that are superinduced by serum in the absence of protein synthesis (Cochran *et al.*, 1983; Hendrickson and Scher, 1983; Kelly *et al.*, 1983; Müller *et al.*, 1984). From this library we have identified a number of cDNA clones of mRNAs that appear within minutes following serum stimulation and then decline rapidly. Purified growth factors and a tumor promoter also stimulate the accumulation of these mRNAs.

Results

Preparation and screening of a cDNA library from stimulated-cell RNA

A λgt10 cDNA library (Huynh *et al.*, 1985) was prepared using poly(A)$^+$ RNA from confluent BALB/c 3T3 cells maintained in 0.5% serum and then exposed for 3 h to medium containing 20% serum (MEM-20) and 10 μg/ml cycloheximide. A library of ~6 × 10^5 independent recombinant phages was obtained and amplified through minimal passage. To screen for cDNAs derived from growth-related RNAs, phages of the library were plated at low density, and replicate plaque lifts were probed with [^{32}P]cDNA prepared from poly(A)$^+$ RNA from either quiescent cells or cells stimulated as described above (Figure 1A). Differentially hybridizing plaques were re-tested as follows. Each of the isolated plaques was replated at low density, and phage

from a plaque chosen at random was inoculated at a single site on an agar plate seeded with the appropriate host. After incubation, replicate plaque lifts were prepared and hybridized to the two probes (Figure 1B). Since the original screening was carried out at low plaque density and thus phages in the secondary plates were nearly homogeneous, repetition of this screening several times was sufficient to examine each clone by random selection. This procedure was used because screening a dishful of secondary plaques often failed to yield hybridization signals, apparently due to the dispersion of limiting amount of probe. Of ~50 000 plaques screened in this manner, 357 were found to hybridize reproducibly to the stimulated cell probe but not to the quiescent cell probe. Each of these clones was twice plaque purified prior to further analysis. In this screening procedure no plaques were found to hybridize preferentially to the quiescent cell probe.

Characterization of cDNA clones

To determine how many different RNAs were represented in this collection of differentially hybridizing cDNA clones, we examined the cloned inserts for sequence homology by cross hybridization. The DNA inserts of some 70 clones were first purified and transferred to a bacterial plasmid vector, pGEM-2. The resulting recombinant plasmids were labeled by nick-translation and hybridized to all phages in the collection. From this analysis we found that four sequence families accounted for 320 of the 357 clones isolated (Table I). At least 10 of the remaining 37 clones represent distinct sequences. Assuming that all cross-hybridizing clones were derived from identical mRNAs, we infer that the number of different mRNAs represented in our set of cDNAs is between 14 and 41; some of these are of very low abundance (see below).

We next investigated the possibility that some of the differentially hybridizing cDNA clones may correspond to RNAs known to be stimulated by serum or growth factors by hybridizing the 357 phage clones with relevant DNA probes available to us. One clone hybridized to a chicken β-actin cDNA (Cleveland et al., 1980); none hybridized to probes for c-fos (Curran et al., 1983), c-myc (Land et al., 1983), ornithine decarboxylase (Kahana and Nathans, 1984), or proliferin (Linzer and Nathans, 1984) under conditions of moderate stringency, nor to probes for abl, erbA, erbB, fes, fgr, fms, met, mos, ras, rel or sis viral oncogenes, nor to B2 repetitive sequences (Krayev et al., 1982). Thus most of the clones appear to correspond to genes not previously known to be associated with cell growth.

RNAs corresponding to selected cDNA clones

For characterization of RNAs corresponding to cDNA clones we selected the five clones noted in Table I. Total RNA from quiescent or serum-stimulated cells was fractionated by formaldehyde-agarose gel electrophoresis and hybridized to the appropriate labeled cDNA. Clones 3CH61, 77, 92, 96 and 134 hybridized to predominant RNAs of 2.7, 3.6, 2.2, 1.6 and 2.8 kb, respectively, detected only in stimulated cells (Figure 2). Oligo(dT)-cellulose-selected RNA was also analyzed with similar results (Figure 2).

To determine the time course of appearance and decay of these RNAs, total cellular RNA was prepared from 3T3 cells at various times after the cells were treated with MEM-20, 3 μg of RNA from each sample was affixed to nitrocellulose and the filters were hybridized to labeled DNA of a given plasmid clone. The patterns of RNAs displayed in RNA blots after resolution by formaldehyde-agrose gel electrophoresis were not changed throughout the time course, nor were they affected by the presence of

Table I. Summary of clones isolated by differential hybridization

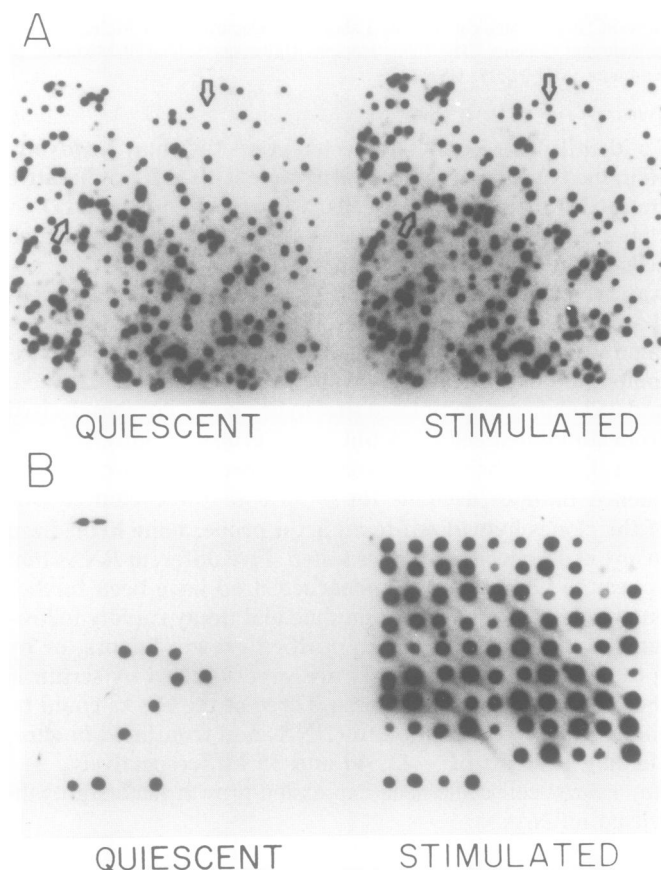| Clone	Number of cross-hybridizing clones	Size of insert (bp)	Size of RNA (kb)	Size of protein product (kd)
3CH61	226	470	2.7	43
3CH134	75	530	2.8	40
3CH77	14	500	3.6	—
3CH92	5	660	2.2	35
3CH96	1	210	1.6	—

Fig. 1. Differential screening of the cDNA library for clones whose RNAs are more abundant in serum-stimulated cells than in quiescent cells. (A) Primary screening: replicas of nitrocellulose filters impressed on agar plates containing phage plaques from the λgt10 cDNA library were hybridized to equivalent amounts of ^{32}P-labeled cDNA probes prepared using poly(A)$^+$ RNA from either quiescent cells or cells stimulated for 3 h with MEM-20 and cycloheximide. Arrows point to phage plaques where differential hybridization signals were observed. (B) Secondary screening: replicas of nitrocellulose filters impressed on agar plates containing phages that showed differential hybridization in the primary screening and were selected and re-screened as described in the text. Each plaque shown represents a different isolate from the initial screening.

cycloheximide (data not shown). As shown in Figure 3A, each of the RNAs appeared within 10−20 min following addition of MEM-20, reached peak levels at ~40−90 min and decayed rapidly over the ensuing 3 h with a half-life of ~30 min (Figure 4). 3CH61 RNA shows a second burst between 6 and 10 h. The amount of each specific RNA in a given sample was quantitated by densitometry and normalized to the level of hybridization of probe to standard amounts of cDNA on the same filter. Each value was then expressed as estimated number of RNA molecules per cell, as presented in Figure 4. The peak levels of the different RNAs varied from ~2 (3CH96) to ~50 (3CH61) copies per cell. Also shown in Figures 3 and 4 are changes in some
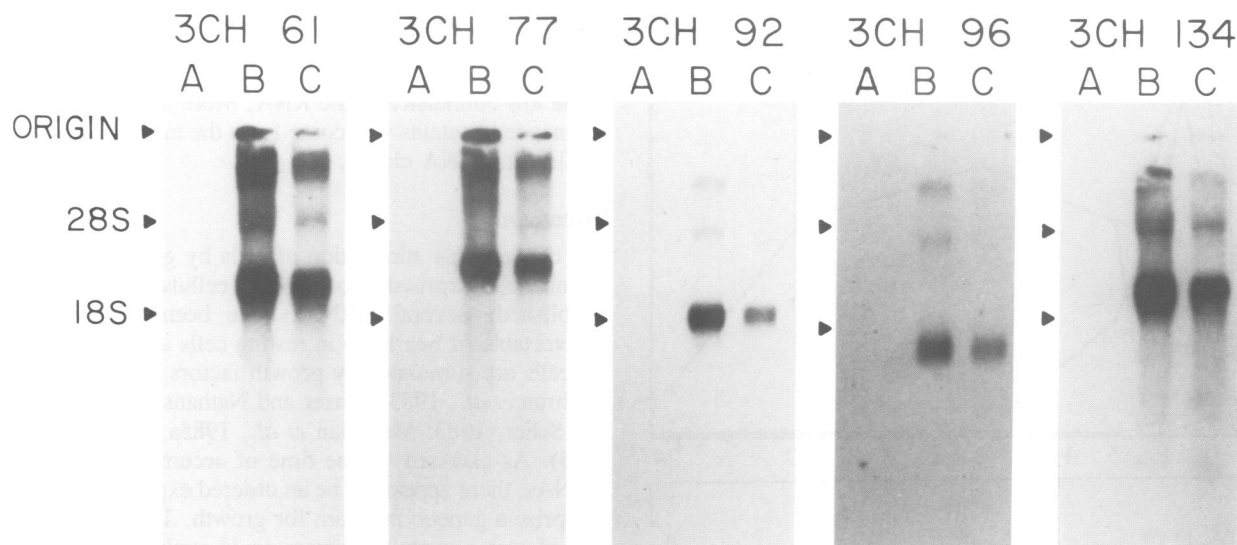
Fig. 2. RNA blot hybridization. 10 μg of total RNA isolated from either quiescent cells (**A**), or cells stimulated with MEM-20 and cycloheximide (10 μg/ml) for 3 h (**B**), or 1 μg of oligo(dT)-cellulose-selected RNA from stimulated cells (**C**) were resolved by formaldehyde-agarose gel electrophoresis. The RNA samples were then transferred to nitrocellulose filters and hybridized to labeled DNA of various clones. Arrows indicate the origin and positions of the 18S and 28S ribosomal RNAs.
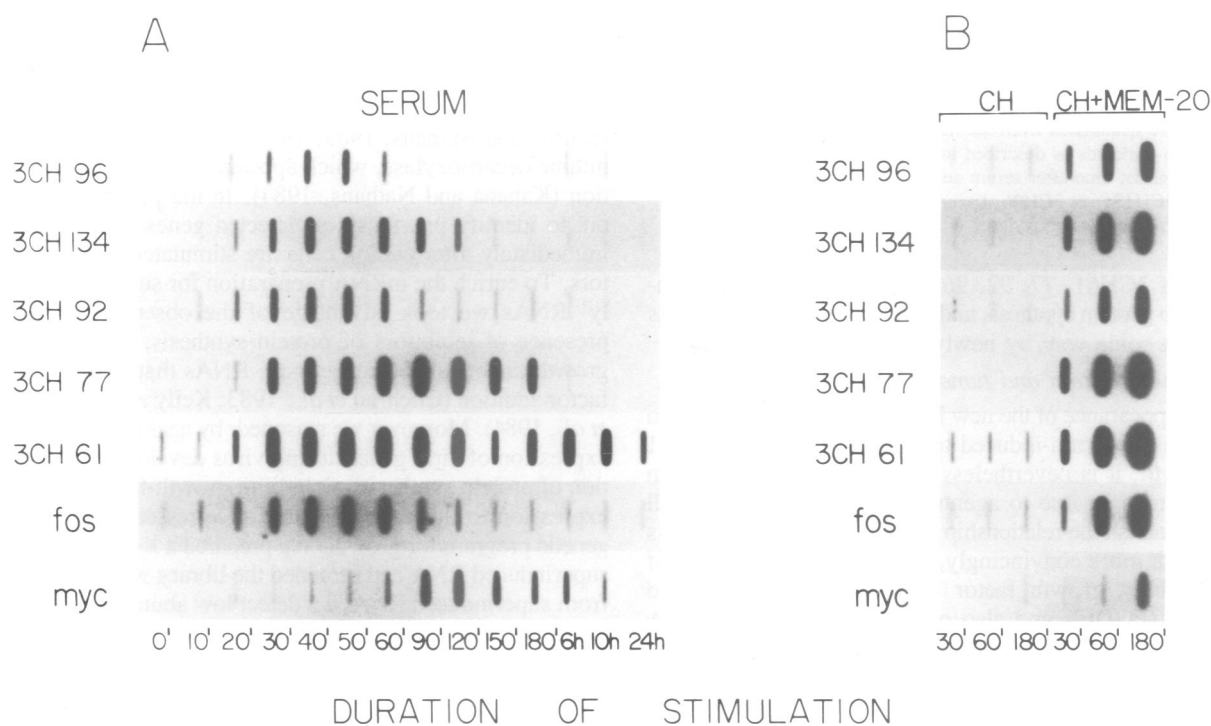

Fig. 3. Accumulation and decay of RNAs stimulated by serum. BALB/c 3T3 cells grown to confluence and brought to quiescence by serum deprivation were stimulated to re-enter the G1 phase with 20% serum (MEM-20). Total RNA (3 μg per sample) isolated from cells after stimulation of the indicated duration were immobilized on nitrocellulose filters and hybridized to labeled DNA of various clones. Accumulation of RNAs in response to stimulation by serum, cycloheximide (CH) or cycloheximide and serum (CH + MEM-20) are shown.

previously characterized growth-related mRNAs, namely those encoding the *fos* and *myc* proteins (Kelly *et al.*, 1983; Greenberg and Ziff, 1984; Kruijer *et al.*, 1984; Müller *et al.*, 1984), ornithine decarboxylase (Kahana and Nathans, 1984) and proliferin (Linzer and Nathans, 1984).

As indicated earlier, one of the reasons for using RNA from cycloheximide-treated cells to prepare a cDNA library was the possible superinduction of 'immediate early' RNAs by serum under these conditions. With the cDNA clones of such RNAs

in hand we could determine whether cycloheximide, either alone or with serum, actually resulted in increased levels of the RNAs. As shown in Figure 3B, cycloheximide alone had little or no effect, whereas cycloheximide plus serum caused a dramatic increase in each of the RNAs tested, including *fos* and *myc* RNAs as previously reported (Kelly *et al.*, 1983; Cochran *et al.*, 1984; Müller *et al.*, 1984). Moreover, high levels of these RNAs persisted for at least 3 h, whereas in the absence of cycloheximide most of these RNAs decayed by 3 h. This result indicates that
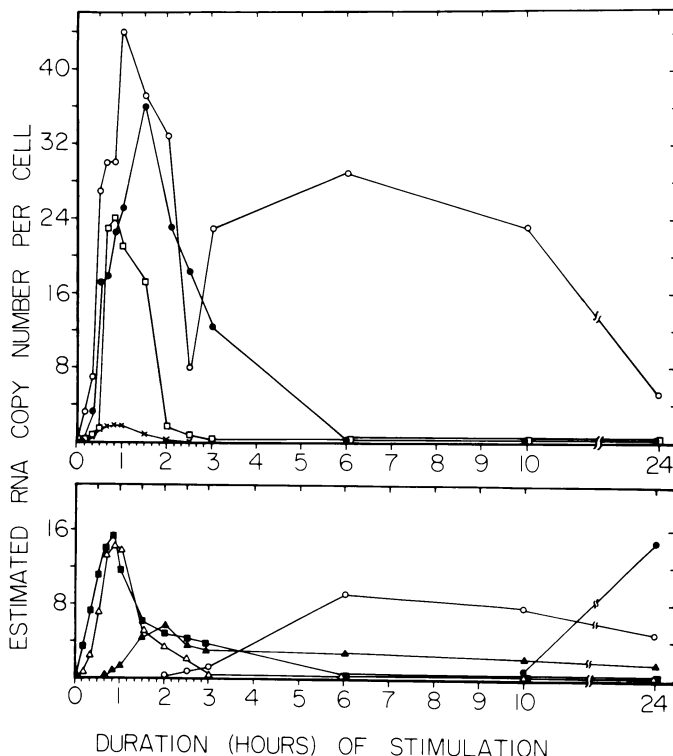
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Fig. 4. Abundance of various RNAs upon serum stimulation. The levels of various RNAs were quantitated from the autoradiograms shown in Figure 3 and normalized to standards as described in Materials and methods. The data are plotted against time after serum addition. Upper panel: ○, 3CH61; ●, 3CH77; □, 3CH134; x, 3CH96, lower panel: ■, 3CH92, △, c-fos; ▲, c-myc; ○, ornithine decarboxylase; ●, proliferin.

the increase of 3CH61, 77, 92, 96 and 134 RNAs is independent of *de novo* protein synthesis and that the level of these RNAs is regulated in some way by newly synthesized protein(s).

Effects of growth factors and tumor promoter

Although the appearance of the new RNAs after addition of serum coincides with the serum-induced transition from the G0 to G1 phase of growth, it is nevertheless possible that the change in RNA species may be due to agents in serum unrelated to cell growth. To establish the relationship between the identified RNAs and cell growth more convincingly, we examined the effects of purified fibroblast growth factor (FGF) and platelet-derived growth factor (PGDF), and also of the tumor promoter TPA (12-O-tetradecanoyl-phorbol-13-acetate), on the level of these RNAs. As shown in Figure 5, FGF and PDGF, to which BALB/c 3T3 cells are known to respond (Stiles, 1983; Gospodarowicz, 1985), and TPA caused an increase in all five RNAs examined. (However, none of these agents showed the marked secondary rise in 3CH61 RNA observed after serum stimulation.) Our interpretation of these results is that the transient increase of these five RNAs during the transition from the G0 to G1 phase of growth is part of the cellular response to mitogenic growth factors.

Translation products of stimulated mRNAs

To begin characterization of the proteins encoded by the mRNAs detected by clones 3CH61, 77, 92, 96 and 134, we purified individual RNAs by hybridization to cDNA clones, carried out *in vitro* translation of the RNAs in the presence of [³⁵S]methionine, and analyzed the protein products by gel electrophoresis. As shown in Figure 6, RNAs hybridizing to clones 3CH61, 92 and

134 directed the synthesis of proteins of ~43, 35 and 40 kd, respectively. Neither 3CH77 nor 3CH96-selected RNA gave a clear translation product; in the case of 3CH96 this may be due to the low abundance of the RNA. More definitive information on encoded proteins will come from the nucleotide sequence of full length cDNA clones.

Discussion

The concept that stimulation of cells by growth factors results in enhanced expression of specific cellular genes is now well established. Several mRNAs have been identified that are undetectable or nearly so in resting cells and appear only after the cells are stimulated by growth factors (Foster *et al.*, 1982; Cochran *et al.*, 1983; Linzer and Nathans, 1983; Hendrickson and Scher, 1983; Matrisian *et al.*, 1985a, 1985b; Levi *et al.*, 1985). As assessed by the time of accumulation of particular mRNAs, there appears to be an ordered expression of genes that comprise a genetic program for growth. The regulated expression of such a genetic program could explain the sequential action of serum growth factors required to stimulate DNA synthesis in resting 3T3 cells (Pledger *et al.*, 1978) and the appearance of specific proteins following growth stimulation of resting cells (Gates and Friedkin, 1978; Riddle *et al.*, 1979; Pledger *et al.*, 1981; Thomas *et al.*, 1981; Croy and Pardee, 1983; Scher *et al.*, 1983).

Our laboratory has previously reported the identification *via* cDNA cloning of an mRNA-encoding proliferin, a prolactin-like protein that appears at ~6 h after serum stimulation of 3T3 cells (Linzer and Nathans, 1983, 1984) and of mRNA encoding ornithine decarboxylase, which appears at ~1−2 h post-stimulation (Kahana and Nathans, 1984). In the present study we set out to identify previously undetected genes that are expressed immediately after resting cells are stimulated with growth factors. To enrich the mRNA preparation for such 'immediate early' RNAs we took advantage of the observation that in the presence of inhibitors of protein synthesis, serum or purified growth factors superinduce those RNAs that appear early after factor addition (Cochran *et al.*, 1983; Kelly *et al.*, 1983; Müller *et al.*, 1984). Moreover we reasoned, by analogy with the ordered expression of viral genes during virus development, that inhibition of protein synthesis might limit growth-factor induced gene expression to those genes that are expressed very early in the genetic program for growth. We prepared a λ cDNA library from superinduced RNA and screened the library with probe prepared from superinduced RNA. To detect low abundance cDNAs during the re-screening of differentially hybridizing phage, each filter contained only one plaque of a given primary isolate, thus concentrating the probe at a single site. Of the 357 clones that reproducibly hybridized with superinduced RNA probe but not with probe from resting cell RNA, there were at least 14 different sequence classes, most of which were found only once in the 50 000 phages screened; these are likely to represent low-abundance, growth-related, mRNAs. Each of the five clones examined in more detail is derived from an mRNA that appears within 10 min of stimulation by serum or growth factor, reaches a peak level in 40−90 min and then rapidly declines, and each is superinduced in the presence of cycloheximide, indicating that new protein synthesis is involved either in repressing transcription and/or RNA processing, or in degradation of this class of mRNAs. Since all five RNAs respond to PDGF, FGF and TPA, they appear to be part of a common response to growth stimuli. Perhaps some of the still uncharacterized clones are derived from RNAs that respond differentially to these agents.
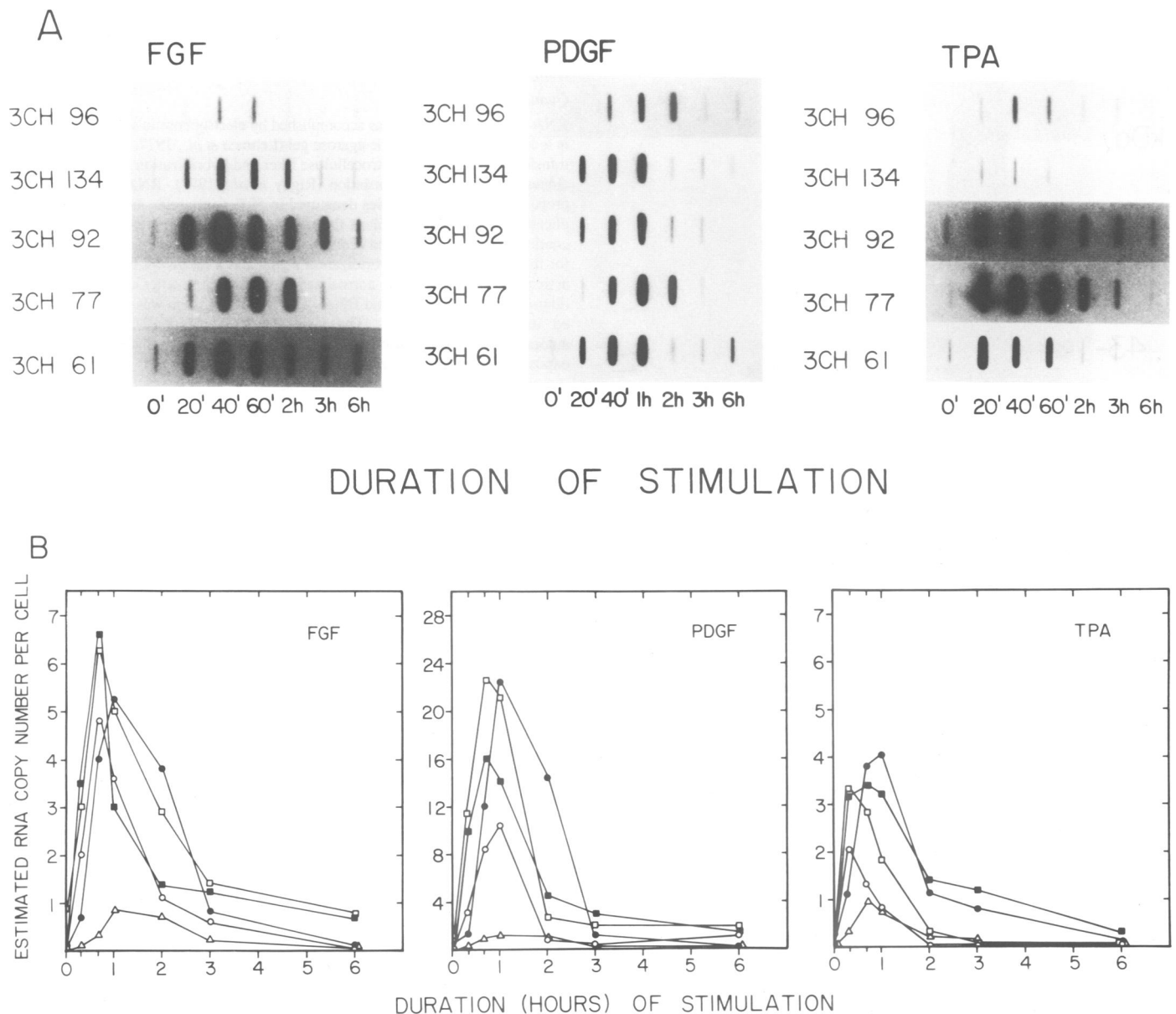
Fig. 5. Response of specific RNAs to defined growth factors or tumor promoter. Cell cultures brought to quiescence by serum deprivation were treated by direct addition of a defined growth factor or tumor promoter to their media, giving final concentrations of FGF at 100 ng/ml, PDGF at 50 ng/ml, or TPA at 100 ng/ml. Total RNA from cells thus treated was isolated at the indicated times after stimulation and immobilized on nitrocellulose filters (3 μg per sample). Hybridization to labeled DNA of various clones shows the time course of accumulation and decay of their corresponding RNAs. The autoradiograms are shown in **A**, and in **B** the normalized data are plotted against time after addition of growth factor or TPA. $\bigcirc$, 3CH61; $\bullet$, 3CH77; $\blacksquare$, 3CH92; $\triangle$, 3CH96; $\square$, 3CH134.

How many growth-related, 'immediate early' RNAs are there? If we include in this class only those RNAs that are induced within ~20 min of exposure to serum or growth factor and do not require protein synthesis for induction, c-*fos* (Greenberg and Ziff, 1984; Müller *et al.*, 1984), c-*myc* (Kelly *et al.*, 1983) and actin (Riddle *et al.*, 1979; Greenberg and Ziff, 1984) are included in this group, and possibly two RNAs previously identified by cDNA cloning (Cochran *et al.*, 1983). We have identified five additional members of this class and several others that are probably in this category. Since we have so far screened only ~1/3 of a complete library (and did not find *fos* or *myc*), the total number of 'immediate early' growth-related genes may be rather large. The likelihood that a large number of mRNAs appear also at subsequent stages following growth stimulation adds to the impression of a complex, tightly regulated genetic program triggered by the interaction of a growth factor with its receptor or by a tumor promoter. Dissection of this program should provide new insight into the regulation of cell growth and the action of oncogenic proteins.

Materials and methods

Cell culture

BALB/c 3T3 clone A31 cells were obtained from the American Type Culture Collection, re-cloned and maintained in MEM-10 [Eagle's minimal essential medium with Earle's salts (GIBCO), glutamine (2 mM), penicillin (10 units/ml), streptomycin (10 units/ml) and 10% fetal bovine serum]. Cells were brought to quiescence by growth in MEM-10 to confluence followed by incubation in MEM-0.5 (0.5% serum) for 3–6 days. For stimulation of quiescent cells the medium was changed to MEM-20 (20% serum), either in the presence or absence
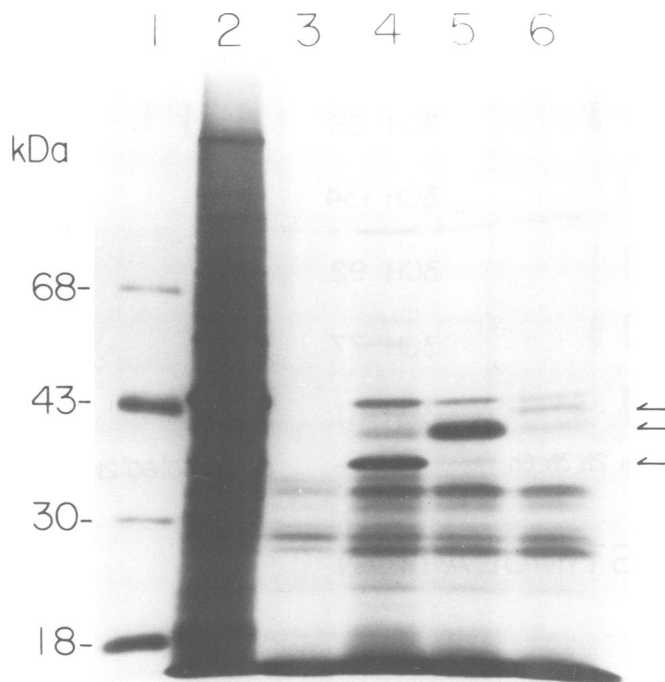
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Fig. 6. Hybrid-selected translation. RNA from cells stimulated with 20% serum (MEM-20) and cycloheximide (10 µg/ml) for 3 h was fractionated by hybrid-selection with a given cDNA clone and the selected RNA was translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Translation products were analyzed by electrophoresis in a 10% NaDodSO$_4$/polyacrylamide gel. **Lane 1,** mol. wt. standards; **lane 2,** translation products of unselected RNA; **lane 3,** endogenous translation products of the reticulocyte lysate; **lane 4,** translation products of clone 3CH92-selected RNA; **lane 5,** translation products of clone 3CH134-selected RNA; **lane 6,** translation products of clone 3CH61-selected RNA. Arrows point to positions where hybrid-selected translation products migrate.

of cycloheximide (10 µg/ml). Stimulation with growth factor or tumor promoter was carried out by direct addition of the agent to the quiescent cell medium.

Enzymes, nucleic acids, growth factors and tumor promoter

All enzymes were purchased from commercial sources. Purified PDGF was obtained from T. Deuel (Deuel *et al.*, 1981); FGF and TPA were from Collaborative Research and Sigma, respectively. Oncogene probes were obtained from the Cell Center Oncogene Collection of the University of Pennsylvania or from the American Type Culture Collection. The chicken β-actin clone was provided by D. Cleveland (Cleveland *et al.*, 1980).

Construction of the cDNA library

Total RNA was isolated from BALB/c 3T3 cells stimulated with MEM-20 and cycloheximide for 3 h using the guanidinium isothiocyanate-cesium chloride precipitation procedure (Chirgwin *et al.*, 1979). Poly(A)$^+$ RNA was purified from total cellular RNA after two cycles of selection on an oligo(dT)-cellulose column (Aviv and Leder, 1972). Double-stranded cDNA was prepared first by reverse transcription of the RNA using an oligo(dT) primer followed by self-primed second strand synthesis catalyzed by *Escherichia coli* DNA polymerase I large fragment (Wickens *et al.*, 1978). Blunt-ends were created by nuclease S1 treatment, the RI sites were modified, and self-complementary *Eco*RI linkers were added. After appropriate restriction, the cDNA was ligated to the vector λgt10 (Huynh *et al.*, 1985) and packaged *in vitro* (Enquist and Sternberg, 1979). The packaged library was plated on *E. coli* C600Δ*Hfl* and amplified through minimal passage in C600. cDNA inserts from clones chosen for further study were transferred to the unique *Eco*RI site of the plasmid vector pGEM-2 (Promega) to facilitate further analysis.

Differential plaque hybridization

The λgt10-cDNA library was plated at low density ($\sim$300 plaques per 9 cm^2 dish) and screened by differential plaque hybridization (Benton and Davis, 1977). Duplicate sets of nitrocellulose filters impressed on agar plates containing recombinant phage plaques were hybridized to ^{32}P-labeled cDNA probe (10^6 d.p.m./ml) prepared from poly(A)$^+$ RNA of quiescent cells or stimulated cells (MEM-20 with cycloheximide, 3-h stimulation). Hybridization conditions were as described (Peden *et al.*, 1982). Differentially hybridizing clones from the initial screen-

ing were selected and re-plated at low density. For re-screening, a single plaque from each secondary plating was transferred in a grid array to an agar plate seeded with host bacteria; duplicate filters were then probed with labeled cDNA prepared from either quiescent or stimulated cell RNA.

Quantitation of RNA

RNA filter hybridization was accomplished by electrophoretic separation of RNA in a denaturing formaldehyde-agarose gel (Lehrach *et al.*, 1977; Goldberg, 1980), transfer of the RNA to a nitrocellulose filter and hybridization to various cDNA clones labeled by nick-translation (Rigby *et al.*, 1977). RNA slot blots were prepared using RNA samples denatured in 50% formamide at 65°C before application to nitrocellulose filters through a slot blotting apparatus. Hybridization conditions were as described (Fellous *et al.*, 1982). To provide a standard curve for the intensities of the hybridization signal and a means by which the specific activities of probe could be normalized, a nitrocellulose filter containing a serial dilution of the cloned plasmid DNA from which the probe was made was included in each hybridization. The signals were quantitated by scanning the autoradiograph using a Joyce-Lobel (MKIIIC) microdensitometer, using several exposures to ensure linear sensitivity.

Hybrid-selected translation

Specific RNAs corresponding to various clones were hybrid-selected as described (Maniatis *et al.*, 1982) and translated in a rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine. The translation products were analyzed by NaDodSO$_4$/polyacrylamide gel (10%) electrophoresis as described (Dreyfuss *et al.*, 1984) followed by fluorography.

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